Contribution of ATM and ATR to the Resistance of Glioblastoma and Malignant Melanoma Cells to the Methylating Anticancer Drug Temozolomide

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Abstract

The major cytotoxic DNA adduct induced by temozolomide and other methylating agents used in malignant glioma and metastasized melanoma therapy is O6-methylguanine (O6-MeG). This primary DNA damage is converted by mismatch repair into secondary lesions, which block replication and in turn induce DNA double-strand breaks that trigger the DNA damage response (DDR). Key upstream players in the DDR are the phosphoinositide 3-kinases ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3 related (ATR). Here, we addressed the question of the importance of ATM and ATR in the cell death response following temozolomide. We show that (i) ATM- and ATR-mutated cells are hypersensitive to temozolomide, (ii) O6-MeG triggers ATM and ATR activation, (iii) knockdown of ATM and ATR enhances cell kill in glioblastoma and malignant melanoma cells with a stronger and significant effect in ATR knockdown cells, (iv) ATR, but not ATM, knockdown abolished phosphorylation of H2AX, CHK1, and CHK2 in glioma cells, and (v) temozolomide-induced cell death was more prominently enhanced by pharmacologic inhibition of CHK1 compared with CHK2. The data suggest that ATM and, even better, ATR inhibition is a useful strategy in sensitizing cancer cells to temozolomide and presumably also other anticancer drugs.

Introduction

Ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3 related (ATR) are both serine/threonine protein kinases important for DNA damage processing. ATM mutated in the homozygous state gives rise to the chromosomal instability syndrome ataxia telangiectasia (A-T, also known as Louis–Bar syndrome; ref. 1). The patients exhibit a strong predisposition to cancer formation and are extremely sensitive to ionizing radiation (2). Homozygous mutation of ATR gives rise to the Seckel syndrome, which is not radiation sensitive, but phenotypically the patients exhibit other peculiarities such as dwarfism (3).

ATM is activated by DNA double-strand breaks (DSB). Active ATM phosphorylates several target proteins, which are involved in cell-cycle control, DSB repair, and the initiation of autophagy and apoptosis. Among them are histone 2AX (H2AX; ref. 4) and CHK2 (5). The latter induces the so-called ATM-CHK2 pathway to block cell-cycle progression. In contrast with ATM, ATR is activated by stalled replication forks (6). Similar to ATM, activated ATR kinase phosphorylates a variety of downstream targets including H2AX (7) and CHK1. CHK1 is then responsible for the ATR-dependent cell-cycle arrest (8). Functionally, the ATM-CHK2 and the ATR-CHK1 pathway are not strictly separated from each other. Thus, CHK1 phosphorylation in response to ionizing radiation is dependent on ATM (9), and the phosphorylation and consequential activation of ATM caused by UV light and stalled replication forks are dependent on ATR (10). Furthermore, CHK2 can be a substrate of ATR in response to ionizing or UV irradiation (11). Clearly there is either considerable crosstalk between the ATM-CHK2 and ATR-CHK1 pathways, or the processing of the primary DNA lesions lead to secondary DNA structures that activate both.

The anticancer drug temozolomide and the model S0,1-methylating agent N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) methylate DNA at 13 different positions (12) forming the main product N7-methylguanine (N7MeG) and the minor adducts N3-methyladenine (N3MeA), N3-methylguanine (N3MeG), and O6-methylguanine (O6-MeG). From these adducts, O6-MeG is clinically most important as it is the main mutagenic, genotoxic, and killing lesion (13). O6-MeG, induced at less than 8% of total methylations by methylating agents, is
Unrepaired O6-MeG triggers apoptosis in a mismatch repair (MMR; ref. 15) and DNA replication (16)-dependent manner. As O6-MeG is a cytotoxic lesion, the level of killing resistance is dependent on the amount of MGMT in the cell and the resynthesize rate of the enzyme. The importance of MGMT in the protection against methylation chemotherapeutics is reflected by the fact that these drugs are used for the treatment of tumors, malignant glioma, and metastatic melanoma, that express low amounts of MGMT (17).

ATM and ATR are activated in a MMR-dependent manner (18). ATR was reported to be activated in the second cell cycle following methylation by methylating agents in synchronized cells (19). In addition, the phosphorylation of CHK1 and p53 upon methylating agent treatment was shown to be dependent on ATM and, along with CHK2, independent of ATM (18). Whether these dependencies were triggered by O6-MeG adducts has not yet been shown. Both ATR and ATM can activate the G2-M cell-cycle arrest following methylation (20, 21). Loss of functional ATM sensitizes mouse fibroblasts to O6-MeG lesions (22). In addition, KU55933, a selective ATM inhibitor, sensitizes glioma cells to temozolomide due to an increased accumulation of DSBs (23). Whether this was a result of O6-MeG adducts in glioma cells was not addressed. These studies differ from earlier studies done on lymphoblastoid and fibroblast cell lines isolated from patients with A-T where no increased sensitivity toward alkylating agents was observed (24, 25).

The present study was conducted to determine the extent of protection that ATM and ATR exert in glioblastoma and malignant melanoma cells against cytotoxicity induced by the critical toxic lesion O6-MeG. The results show that ATR plays a dominant role in causing drug resistance compared with ATM that contributes to a lesser extent. The data also show that ATR activates H2AX, CHK1, and CHK2 in response to O6-MeG and that the inhibition of CHK1 or CHK2 sensitizes cells to this DNA lesion. As the results were obtained in glioblastoma and malignant melanoma cells, they will be of interest for these tumor types during therapy.

Materials and Methods

Cell lines and culture conditions

All the cell lines used in this study are of human origin. Three SV40-immortalized fibroblast cell lines [GM637 (wt), ATs4 (ATM mt), and GM05849 (ATM mt)], two EBV-immortalized lymphoblastoid cell lines [AG09387 (wt) and DK0064 (ATR mt)], one melanoma cell line (D03), and one glioma cell line (LN229). The GM637 cells were described in ref. 26, whereas the ATs4 cells were described in ref. 27. The line, GM05849, was from the Coriell Institute (Camden, New Jersey). The lymphoblastoid cell lines, AG09387 and DK0064, were a gift from M. O’Driscoll (Human DNA Damage Response Disorders Group, Genome Damage and Stability Center, University of Sussex, Brighton, United Kingdom). The melanoma cells D03, described in ref. 28, were a gift from C.W. Schmidt (Immunotherapy Laboratory, QIMR Berghofer Medical Research Institute, Brisbane, QLD, Australia), and the glioblastoma cell line LN229, described in ref. 29, was from M. Weller (Department of Neurology, University Hospital Zürich, and Neuroscience Center Zürich, University of Zürich, Zürich, Switzerland). All the lines were carefully characterized in the laboratory they originated from, displayed the expected phenotype, but were not reauthenticated in our laboratory. All of them, except for LN229, were cultivated in RPMI containing 10% fetal calf serum (FCS) and penicillin/streptomycin (10 U/mL, 0.1 mg/mL). LN229 was cultured in Dulbecco’s Modified Eagle Medium containing 10% FCS and penicillin/streptomycin (10 U/mL, 0.1 mg/mL). All cells were kept in a humid atmosphere with 5% CO2 at 37°C. LN229-MGMT-12 is a cell line stably overexpressing MGMT, the generation of which has been described previously (30). LN229-MGMT-12 was maintained in medium containing 0.5 mg/mL G418. G418 was omitted from the medium during experiments. To maintain genome stability, the cell cultures were frequently replaced by new stocks stored in liquid nitrogen. Doubling times for all cell lines except GM05849 and the MGMT transfectants are shown in Supplementary Table S1.

Drug treatment

Cells were exposed to the genotoxic agents MNNG (Sigma-Aldrich) and temozolomide (Schering-Plough) by adding the drugs once to the medium. Unless otherwise stated, 1 hour before the addition of MNNG or temozolomide, 10 μmol/L O6-benzylguanine (O6-BG, Sigma-Aldrich) was added to the medium to inactivate MGMT. In the experiment with LN229-MGMT-12, the O6-BG treatment was repeated 24 and 48 hours after temozolomide treatment (2.5 μmol/L). Transfection was conducted during experiments. To maintain genome stability, the cell cultures were frequently replaced by new stocks stored in liquid nitrogen. Doubling times for all cell lines except GM05849 and the MGMT transfectants are shown in Supplementary Table S1.

Transient transfection of the tumor cells with siRNA targeting ATM- and ATR-mRNA

Transient protein knockdown was accomplished by using siGENOME SMARTpool siRNAs obtained from Thermo Scientific. One nonsense-siRNA and siRNAs against ATM and ATR were used in the concentration of 10 nmol/L. Transfection was conducted with the transfection reagent Lipofectamine RNAiMax (Invitrogen) according to the manufacturer’s protocol. Protein knockdown was verified by Western blot analysis 48 hours after transfection. Cells were treated with the indicated doses of temozolomide 24 hours after transfection.
Stable transfection of the fibroblasts with human MGMT cDNA

Exogenous MGMT expression was accomplished by cotransfection of the wild-type (wt) and ATM-mutated fibroblasts with 0.9 μg of the expression vector pSV2 containing the human MGMT cDNA, which has been described previously (13), and 0.1 μg of the pSV2neo plasmid for selection. For transfection, the Effectorne transfection kit (QIAGEN) was used. After transfection, cells were selected with 0.5 mg/mL G418 (Sigma-Aldrich). Appearing clones were then picked and checked by Western blot analyses for MGMT expression. Transfected cells were maintained in medium containing 0.5 mg/mL G418. G418 was omitted from the medium during experiments.

MTT assay

The MTT assay was done using the Thiazolyl Blue Tetrazolium Bromide obtained from Sigma-Aldrich. Briefly, cells growing in suspension were harvested and diluted in medium containing 1.67 mg/mL MTT solution. After completion of the MTT reaction, medium was removed and cells were incubated for 15 minutes in 0.04 mol/L HCl/isopropanol. Absorbance was measured using a photometer (Tecan) with a wavelength of 570 nm and a reference wavelength of 650 nm.

Annexin V/PI double staining

For Annexin V/PI double staining, cells were treated as described in the previous section, further incubated, harvested, and resuspended in 50 μL Annexin V-binding buffer (10 mmol/L HEPES, 140 mmol/L NaCl, 2.5 mmol/L CaCl2, 0.1% bovine serum albumin, pH 7.4). Of note: 2.5 μL Annexin V-FITC reagent (Miltenyi Biotec) was added, and cells were incubated on ice for 15 minutes. Afterwards, DNA in the cells was stained with 1 μg/mL propidium iodide (PI) and analyzed with the FACS Calibur or FACS Canto II (BD Biosciences) using the CellQuest Pro or the FACS Diva (BD Biosciences) and WinMDI software (J. Trotter).

BER assay

The base excision repair (BER) assay was conducted as described previously (31). Synthetic oligonucleotides purchased from Eurofins and 32P-labeled ATP obtained from Perkin Elmer were used.

Alkaline comet assay

At indicated time points after drug addition, cells were harvested and imbedded in low melting point agarose on glass slides. Cells were lysed for 60 minutes with cold alkaline lysis buffer (2.5 mol/L NaCl, 100 mmol/L EDTA, 10 mmol/L Tris, 1% sodium lauryl sarcosinate, 1% Triton X-100, 10% dimethyl sulfoxide, pH 10). Before electrophoresis for 15 minutes at 25 V and 300 mA, cells were incubated in cold electrophoresis buffer (300 mmol/L NaOH, 1 mmol/L EDTA, pH > 13) for 20 minutes. Subsequently, the slides were neutralized (0.4 mol/L Tris, pH 7.5) and dehydrated with 100% ethanol. Dried slides were stained with 50 μg/mL PI and analyzed using the microscope (Nikon Microphot-FXA). Comet scores were measured using the Komet v4.0 software (Kinetic Imaging).

Immune fluorescence

At the indicated time points following drug addition, cells grown on glass cover slips were fixed with either 4% formaldehyde or with 70% methanol/30% aceton and blocked with goat serum. Then cells were incubated overnight with anti-pH2AX primary antibody (1:1000, Millipore), washed with PBS, and incubated with antimouse secondary antibody conjugated to Cy3 (1:600, Jackson Immuno Research). Cells were stained with 1 μmol/L To-Pro-3 (Invitrogen) and mounted on glass slides in Vectashield mounting medium (Vector Laboratories) and sealed using nail varnish. Microscopy was conducted by laser scanning microscope (Carl Zeiss) with the ZEN 2008 software (Carl Zeiss).

Preparation of cell extracts

Whole-cell protein extracts for Western blot analyses were prepared by two different protocols as described (32, 33). Protein concentration was determined with the Bradford assay (34).

Western blotting

Western blot analysis was conducted as published (35). The following primary antibodies were used: Anti-ATM (Cell Signaling Technology), Anti-ATR (Cell Signaling Technology), anti-cleaved caspase-7 (Cell Signaling Technology), anti-PARP-1 (BD Transduction Laboratories), anti-MGMT (raised in the laboratory of B. Kaina), anti-pChk1 (Ser345; Cell Signaling Technology), anti-pChk2 (Thr68; Cell Signaling Technology), anti-ERK2 (Santa Cruz Biotechnology), and anti-Talin1 (Cell Signaling Technology). Horseradish peroxidase-coupled and fluorophor-coupled secondary antibodies (Amersham Biosciences and LI-COR Biosciences) were used for detecting the primary antibodies. Proteins were visualized using a chemiluminescence detection reagent (GE Healthcare) or the Odyssey system (LI-COR Biosciences).

Statistical analysis

For statistical analysis, the unpaired t test was conducted using the Prism v3 software (GraphPad Software). Values presented in graphs are the means of at least three independent experiments.

Results

ATM and ATR protect from cell death induced by methylating agents

To determine whether ATM and/or ATR plays a role in the protection of cells against methylating agent-induced cell death, cell pairs wt and mutated in ATM or ATR were compared in their response to methylating agents. Comparing the ATM and ATR protein levels in the cell lines
(GM637 wt and ATs4 ATM mt, and AG09387 wt and DK0064 ATR mt, respectively), it is obvious that the mutant cell lines express lower levels of the respective proteins than the wt cells (Fig. 1A). For all subsequent experiments, unless stated otherwise, cells were pretreated with the MGMT inhibitor O6-BG to exclude any variability caused by differences in MGMT expression between the lines. The ATM- (Fig. 1B) and the ATR-defective cell lines (Fig. 1C) were significantly more sensitive to temozolomide than the corresponding wt. To substantiate these cytotoxicity data, the cell pairs were subjected to the Annexin V/PI assay that determines apoptosis and necrosis after a genotoxic insult. Again, ATM- (Fig. 1D) and ATR (Fig. 1E)-defective cells were significantly more sensitive to temozolomide than the corresponding wt. Data for the combined values (the sum of apoptosis and necrosis) are presented in Fig. 1; for the actual apoptosis and necrosis levels see Supplementary Table S2. We should note that the ATR mt cells retain residual ATR activity (3), which might explain the small difference in TMZ sensitivity compared to ATM mt cells. We checked another ATM-mutated cell line (GM05849 ATM mt) as to its temozolomide sensitivity, which again proved to be more sensitive than the wt (Fig. 1D). For further verification, the activation of caspase-7 (cleaved form) and PARP-1 cleavage was probed in the wt and

Figure 1. Induced cellular toxicity following temozolomide and MNNG in wt, ATM, and ATR-mutated cells. A, ATM and ATR expression determined by Western blot analysis of whole-cell protein extracts of fibroblasts and lymphoblastoid cells. B and C, cell viability analyzed with the MTT assay 72 hours after exposure to temozolomide with indicated concentrations in fibroblasts (B) and lymphoblastoid cells (C). D and E, induced overall cell death determined with flow-cytometric analysis of Annexin V/PI double-stained cells 72 hours after temozolomide treatment with indicated concentrations in human fibroblasts (D) and lymphoblastoid cells (E). The overall cell death was calculated by combining the apoptotic and necrotic fraction. F, cleavage of caspase-7 and PARP-1 after treatment with 10 μmol/L MNNG investigated by Western blot analysis of whole-cell protein extracts of fibroblasts. To inactivate MGMT, cells were cotreated with 10 μmol/L O6-BG in all experiments. ERK2, extracellular signal-regulated kinase-2, used as loading control. *, \( P < 0.05; \) **, \( P < 0.005; \) and ***, \( P < 0.0001. \)
ATM-mutated fibroblasts; this time the cell pair was treated with the model methylating agent MNNG (Fig. 1F). The ATM-defective cells showed clearly more caspase-7 activation along with PARP-1 cleavage than the wt cells. Collectively, these data show that both ATM and ATR protect cells against cell death triggered by methylating agents.

ATR, and not ATM, plays the dominant role in protecting malignant glioma and melanoma cells against temozolomide

As temozolomide is used as first line in the therapy of malignant glioma and metastasized melanoma, would the loss of ATM and/or ATR be of benefit in sensitizing these highly refractory cancers? To this end, ATM and ATR were transiently knocked down in glioma (LN229) and melanoma (D03) cells (G). Cells were transfected transiently with 10 nmol/L siRNAs. Temozolomide treatment was conducted 24 hours after protein knockdown. MGMT was inactivated by cotreatment of the cells with 10 μmol/L O6-BG. To keep the overall siRNA concentration constant, cells transfected with 20 nmol/L control siRNA were used to compare with the cells that received the ATM/ATR double knockdown.

O6-methylguanine triggers the activation of ATM and ATR

In all previous experiments, MGMT was inactivated to be able to determine the full extent of the contribution that O6-MeG has on cell death induced by methylating agents. However, along with O6-MeG, methylating agents such as temozolomide also induce N-methylpurines, and both of these types of lesions can exert cytotoxicity. Therefore, to determine whether ATM and ATR protect against cell death triggered by O6-MeG or the N-methylpurines, cell lines that do not express adequate MGMT levels were stably transfected with a plasmid containing the cDNA of MGMT. Transfection gave rise to an increased MGMT protein level in the fibroblast lines, GM637 wt and ATs4 ATM mt, and in the glioblastoma line LN229 (Fig. 3A). For the lymphoblastoid ATR cell pair, transfection with...
MGMT was unnecessary as the lines AG09387 wt and DK0064 ATR mt contained similarly high MGMT protein levels (Fig. 3A). MGMT assays were conducted to verify the activity of MGMT in the lymphoblastoid ATR cell pair and the increase in MGMT activity in the MGMT-transfected cells (data not shown).

Having established an experimental system where cells are protected by MGMT against cytotoxicity caused by O6-MeG, we were able to determine whether ATM and ATR protect against O6-MeG or the N-methylpurines. In the MGMT-expressing ATM mt (Fig. 3B) and the ATR mt (Fig. 3C) lines, methylating agents were unable to exert any significant cytotoxicity, whereas inactivation of MGMT with O6-BG restored their extreme sensitivity toward methylating agents. This experimental approach was extended to the glioma cell system. Similar to what was observed in the ATM and ATR mutants, overexpression of MGMT completely abolished temozolomide-induced cytotoxicity, even when ATM and ATR were downregulated by knockdown (Fig. 3D). Again, when
the critical toxic lesion induced by O6-methylating agents caused a decrease (Fig. 4E). These data show that O6-meG caused an increase in foci, whereas knockdown of ATR

Knockdown of ATM and ATR had opposing effects on nolfluorescence (Fig. 4D for representative images). Knockdown and temozolomide treatment using immu-
determined in the glioma cell line following ATM or ATR

ATR and that ATM knockdown has an effect on the
rest on the formation of the minor DNA adduct O6-
MeG, which is converted by DNA replication and MMR into DSBs (39) that trigger apoptosis, necrosis, and autophagy (16, 40). Temozolomide has been shown to induce the DNA damage response (DDR), which rests on the activation of the phosphoinositide 3-kinases, ATM, ATR (18), and presumably also DNA-PKcs (41), and phosphorylation of downstream targets such as histone 2AX, CHK1, and CHK2 (16, 18). This finally leads to activation of the apoptotic pathway in malignant glioma (41) and melanoma cells (33, 42). Although it is likely that all of these responses are triggered by O6-MeG, a systematic study is lacking as to the importance of ATM and ATR in the O6-MeG-triggered temozolomide response.

Here, we addressed the question of the contribution of ATM and ATR in the killing response of glioblastoma
Figure 4. BER capacity of wt and ATM-mutated fibroblasts and γH2AX foci formation in glioblastoma cells. A, representative gel of the in vitro BER assay. The cleavage and reconstitution of a synthetic DNA oligonucleotide containing a single apurinic site in the presence of whole-cell protein extracts is shown as a function of time. B, quantification of the relative BER capacity by comparing the intensity of the restored DNA oligonucleotide with the control at indicated time points. C, detection of DNA SSBs by the alkaline comet assay following a 1-hour pulse of 15 μmol/L MNNG. D, images of representative glioblastoma cell nuclei, which were investigated by immunofluorescence 48 hours following 1 μmol/L temozolomide treatment. Twenty-four hours before drug treatment, cells were transfected transiently with siRNAs (control, ATM, or ATR) with a final concentration of 10 nmol/L. To deplete MGMT, cells were cotreated with 10 μmol/L O6-BG. To-Pro-3–stained cell nuclei are shown in blue, γH2AX foci in red. E, quantification of γH2AX foci shown in C. **, P < 0.05; and $$$, P < 0.005.
and malignant melanoma cells to temozolomide, using different cell models. First, we show that cells obtained from patients with A-T and Seckel syndrome are hypersensitive to temozolomide, responding with a prompt cell death induction. The ATM data confirm a previous study with mouse ATM knockout cells that showed that ATM knockout cells are more sensitive than the corresponding wt to the O6-methylating agent MNNG (22). In ATR-mutated cells, the O6-MeG response has not been studied to date.

Having shown that both ATM and ATR determine the resistance of cells to temozolomide, we extended the study to glioblastoma and malignant melanoma cells, using the well-characterized lines LN229 (29) and D03 (28), respectively. This was accomplished by downregulation of ATM or ATR in these cells and determining the impact on their sensitivity to temozolomide. Knockdown of either ATM or ATR greatly enhanced the sensitivity of LN229 and D03 cells to temozolomide, with a significantly stronger sensitizing effect if ATR was downregulated. Simultaneous downregulation of ATM and ATR had no additional effect on the killing response. The data indicate that ATM and, even more, ATR, are targets for improving the anticancer effect of temozolomide. This study revealed that temozolomide treatment results in the activation of both CHK2 and CHK1. Because MGMT expression nearly completely abrogated CHK2 and CHK1 activation, we conclude that the specific DNA damage O6-MeG is responsible for triggering this response. Therefore, we wondered whether inhibition of either CHK2 or CHK1 is effective in enhancing the killing effect of temozolomide. Pharmacologic inhibition of CHK1 and to some extent also CHK2 enhanced the cell killing response to temozolomide. This enhancement was completely abolished if MGMT was expressed in the cells, which substantiates the model that O6-MeG DNA adducts are the primary lesions triggering the ATM-ATR-CHK1-CHK2 pathways.

According to the "classical" model, ATM is triggered by free DSBs, whereas ATR becomes activated by blocked replication forks resulting from adducts induced by UV light and chemical DNA-damaging agents (45, 46). We, therefore, supposed that ATM does not play a role in the temozolomide-induced DDR, as the drug does not directly induce free DSBs. However, O6-MeG/thymine mispairs processed by MMR cause secondary lesions that severely block replication in the second cell cycle (19), which in turn lead to the formation of DSBs (19). This scenario supports the model that blocked replication forks activate ATR, and that ATM is activated as a secondary consequence of replication inhibition giving rise to DSB formation at blocked replication forks. We should note that ATR and ATM do not exclusively activate CHK1 and CHK2, respectively.
but that there is crosstalk between both pathways (9–11). Therefore, both CHK1 and CHK2 activation in glioma and melanoma cells following temozolomide treatment is likely mediated by ATR, which was shown here to be the case.

Interestingly, we observed that knockdown of ATM enhanced the amount of temozolomide-induced γH2AX foci, whereas knockdown of ATR reduced the γH2AX foci level. We explain this finding on the basis of the supposition that ATR is the primary kinase activated by O6-MeG-induced secondary lesions that trigger the phosphorylation of H2AX. Therefore, lack of ATR abolishes H2AX phosphorylation. In contrast, ATM might be acting as a repair stimulator of DSBs resulting from O6-MeG/T mispairs, without being required for the initial recognition of stalled replication forks, which arise after initiation of O6-MeG–induced futile MMR cycles.

If ATR and ATM have an impact on the sensitivity of glioblastoma and malignant melanoma cells to O6-methylating drugs (temozolomide, and also dacarbazine and procarbazine that need metabolic activation by liver cytochrome P450), it is pertinent to conclude that the expression level of these kinases as well as their mutation status is important for the therapeutic response. Because MGMT abolishes the increased sensitivity given by the impaired function of ATM or ATR, the therapeutic benefit is limited to patients with tumors that lack MGMT expression. In malignant glioma specimens, a reduced level of expression (47), variable expression (48), and high expression (49) of ATM has been reported. In metastasized melanoma, high ATM expression compared with normal cells was reported (50). With respect to ATR, downregulation of expression was found in malignant gliomas compared with normal brain tissue (51). About the expression of checkpoint kinases, glioblastoma (52), but not sporadic glioma (53), was shown to carry mutations in CHK2. It was also shown that CHK1 and CHK2 expression was downregulated by promoter hypermethylation in glioma (51). Mutations in genes encoding both proteins were also found in metastasized melanoma cells (54).

ATM expression was related to the therapeutic outcome in patients with glioblastoma following temozolomide treatment in combination with ionizing radiation (55). CHK2 gene polymorphism also correlated with prognosis of patients with glioblastoma receiving radiochemotherapy (56). Most studies pertained
to the response of glioblastoma cells to radiation therapy. Thus, loss of ATM and CHK2 was reported to provoke radioresistance in a mouse glioma model (57), whereas pharmacologic inhibition of CHK1 and CHK2 reverted radioresistance in glioma cells in vitro (58). Because the radioresponse of cells, including the activation of death pathways, is clearly different from chemical agents, translation of these data to temozolomide-triggered responses is not possible. Previous studies revealed that CHK1 inhibition increases temozolomide cytotoxicity (59), which is in line with our data.

This is the first study in which the role of ATR and ATM, CHK1 and CHK2 triggered by a specific DNA damage, O6-MeG, in the killing response of malignant glioma and melanoma cells to temozolomide was investigated comparatively. We identified both pathways to be involved, with a dominance of the ATR pathway. Therefore, inhibition strategies of this pathway (59, 60) together with inactivation of MGMT (61) and homologous recombination mediated by Rad51 and BRCA2 (30) might be reasonable strategies for improving the effectiveness of treatment of malignant glioma and melanoma cells with temozolomide and other O6-methylating treatment modalities.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: M. Eich, W.P. Roos, B. Kaina
Development of methodology: M. Eich, W.P. Roos, T. Nikolova
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Eich, T. Nikolova, B. Kaina
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): B. Kaina
Study supervision: B. Kaina

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